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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Horvitz et al.	Confirmation No.:	7921
Serial No.:	10/661,398	Art Unit:	1636
Filed:	September 12, 2003	Examiner:	Catherine S. Hibbert
		Customer No.:	

Title: RB PATHWAY AND CHROMATIN REMODELING GENES THAT ANTAGONIZE LET-60 RAS SIGNALING

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DECLARATION OF H. ROBERT HORVITZ, PH.D. UNDER 37 C.F.R. § 1.132

1. I am a professor at the Massachusetts Institute of Technology, the Assignee of the above-captioned patent application. I am an inventor of claimed subject matter examined in the present application. I have over 30 years of research experience in the fields of genetics and molecular biology. Enclosed is a document showing a list of my positions and honors, and a list of select articles from the numerous scientific articles that I have authored or co-authored (Exhibit A).

2. Prior to the filing date of the application, Ras family genes and Rb family genes were known to be structurally and functionally conserved between *C. elegans* and

mammals, and expressed in a variety of cell types. Based on the known conservation of Ras family and Rb family genes in numerous cell types, and the Examples in the specification of the present application, I would have expected, at the time of filing, one skilled in the art of molecular biology to have been capable of using the methods claimed in the present application that utilize a cell having a loss of function mutation in *mep-1*, *lin(n3628)*, *lin(n4256)*, or *lin-65*, and a second loss of function in a Class A synMuv gene, to identify candidate compounds for treating neoplasia using any one of a variety of cell types including various *C. elegans* and mammalian cells.

3. All statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true; and further these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

7/29/08
Date

H. Robert Horvitz, Ph.D

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors.
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NAME	POSITION TITLE
H. Robert Horvitz, PhD	Professor of Biology/PI
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EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
M.I.T., Cambridge, MA	S.B.	1968	Mathematics
M.I.T., Cambridge, MA	S.B.	1968	Economics
Harvard University, Cambridge, MA	M.A.	1972	Biology
Harvard University, Cambridge, MA	Ph.D.	1974	Biology
M.R.C. Molecular Biology, Cambridge, UK	----	----	Biology

A. Positions and Honors.

Positions and Employment

1978-1981	Assistant Professor, MIT Dept. of Biology, Cambridge, MA
1981-1986	Associate Professor, MIT Dept. of Biology, Cambridge, MA
1986-present	David H. Koch Prof. of Biology, MIT Dept. of Biology, Cambridge, MA
2000-present	Howard Hughes Medical Institute Investigator
1989-present	Mass. Genl. Hosp., Boston, MA, Division of Medicine (Geneticist)
1989-present	Mass. Genl. Hosp., Boston, MA, Division of Neurology (Neurobiologist)

Honors

Genetics Society (U.K.), Mendel Medal, 2007; Eli Lilly Lecturer Award, 2007; James R. Killian Faculty Achievement Award (MIT), 2006; Honorary D.Sc., Pennsylvania State University, 2006; Centennial Medal, Harvard University, 2005; Alfred G. Knudson Award, National Cancer Institute, 2005; Honorary D.Sc., Cambridge University, 2004; American Philosophical Society, Member, 2004; Honorary M.D., University of Rome, 2004; U.S. Institute of Medicine, Member, 2003; Nobel Prize in Physiology or Medicine, 2002; Peter Gruber Fndn. Genetics Prize, 2002; American Cancer Society Medal of Honor, 2002; Wiley Prize in Biomedical Sciences, 2002; Genetics Society of America Medal, 2001; Bristol-Myers Squibb Award in Neuroscience, 2001; Charles-Leopold Mayer Prize (French Academy of Sciences), 2002; Louisa Gross Horwitz Prize for Biology or Biochemistry, 2000; March of Dimes Prize in Develop. Biol., 2000; Paul Ehrlich and Ludwig Darmstaedter Prize (Frankfurt, Germany), 2000; Gairdner Fndn. Intl. Award (Toronto, Canada), 1999; General Motors Cancer Research Fndn., Alfred P. Sloan, Jr., Prize, 1998; Passano Award, 1998; Rosenstiel Award (Brandeis Univ.), 1998; Amer. Acad. Microbiology, Fellow, 1997; Ciba-Drew Award for Biomedical Science, 1996; Charles A. Dana Award for Pioneering Achievements in Health, 1995; Hans Sigrist Award (Univ. Bern, Switzerland), 1994; Amer. Acad. Arts & Sciences, Fellow, 1994; V.D. Mattia Award (Roche Inst. of Molecular Biology), 1993; U.S. Natl. Acad. Sci., Member 1991; Amer. Assoc. Adv. Science, Fellow, 1989; U.S. Steel Fndn. Award in Molec. Biol. (U.S. Natl. Acad. Sci.), 1988; Warren Triennial Prize of Mass. Genl. Hosp., 1986; Spencer Award in Neurobiology (Columbia Univ.), 1986.

B. Selected peer-reviewed publications (in chronological order).

Chen, F., Hersh, B., Conradt, B., Zhou, Z., Riemer, D., Gruenbaum, Y. and Horvitz, H.R. (2000). Translocation of *C. elegans* CED-4 to nuclear membranes during programmed cell death. Science 287, 1485-1489.

Reinhart, B.J., Slack, F.J., Basson, M., Pasquinelli, A.E., Bettinger, J.C., Rougvie, A.E., Horvitz, H.R. and Ruvkun, G. (2000). The 21-nucleotide *let*-7 RNA regulates developmental timing in *Caenorhabditis elegans*. Nature 403, 901-906.

Reddien, P.W. and Horvitz, H.R. (2000). CED-2/CrkII and CED-10/Rac control phagocytosis and cell migration in *Caenorhabditis elegans*. Nature Cell Biology 2, 131-136.

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Ceol, C. and Horvitz, H.R. (2001) *dpl-1* DP and *efl-1*E2F act with *lin-35* Rb to antagonize Ras signaling in *C. elegans* vulval development. Molec. Cell 7, 461-473.

Reddien, P., Cameron, S. and Horvitz, H.R. (2001) Phagocytosis promotes programmed cell death in *C. elegans*. Nature 412, 198-202.

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Jager, S., Schwartz, H. Horvitz, H.R., and Conradt, B. (2004) The *Caenorhabditis elegans* F-box protein SEL-10 promotes female development and may target FEM-1 and FEM-3 for degradation by the proteasome. Proc. Natl. Acad. Sci. U.S.A. 101, 12549-12554.

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Alkema, M., Hunter-Ensor, M., Ringstad, N. and Horvitz, H.R. (2005) Tyramine functions independently of octopamine in the *Caenorhabditis elegans* nervous system. Neuron 46, 247-260.

Lu, J., Getz, G., Miska, E., Alvarez-Saavedra, E., Lamb, J., Peck, D., Sweet-Cordero, A., Ferrando, A., Downing, J., Jacks, T., Horvitz, H.R. and Golub, T. (2005) MicroRNA expression profiles classify human cancers. Nature 435, 834-848.

Wienholds, E., Kloosterman, W., Miska, E., Alvarez-Saavedra, E., Berezikov, E., de Bruijn, E., Tolstrup, N., Horvitz, H.R., Kauppinen, S., and Plasterk, R. (2005) MicroRNA expression in zebrafish embryonic development. Science 309, 310-311.

Abbott, A., Alvarez-Saavedra, E., Miska, E., Lau, N., Bartel, D., Horvitz, H.R. and Ambros, V. (2005) The *let-7* microRNA family members *mir-48, mir-84* and *mir-241* function together to regulate developmental timing in *Caenorhabditis elegans*. Develop. Cell 9, 403-414.

Frank, C. A., Hawkins, N., Guenther, C., Horvitz, H.R., and Garriga, G. (2005) *C. elegans* HAM-1 positions the cleavage plane of certain asymmetric neuroblast divisions. Develop. Biol. 284, 301-310.

Davison, E., Harrison, M., Walhout, A., Vidal, M and Horvitz, H.R. (2005) *lin-8*, which antagonizes *Caenorhabditis elegans* Ras-mediated vulval induction, encodes a novel nuclear protein that interacts with the LIN-35 Rb protein. Genetics 171, 1017-1031.

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Berdichevsky, A., Viswanathan, M., Horvitz, H.R. and Guarente, L. (2006) *C. elegans* SIR-2.1 interacts with 14-3-3 proteins to activate DAF-16 and extend life span. Cell 125, 1165-1177.

Andersen, E., Lu, X. and Horvitz, H.R. *C. elegans* ISW1 and NURF301 antagonize an Rb-like pathway in the determination of multiple cell fates. (2006) Development 133, 2695-2704.

Su, C.-W., Tharin, S., Jin, Y., Wightman, B., Spector, M., Meili, D., Tsung, Rhiner, C., Bourikas, D., Stoeckli, E., Garriga, G., Horvitz, H.R., and Hengartner, M. The short coiled-coil domain-containing protein UNC-69 cooperates with UNC-76 to regulate axonal outgrowth and normal presynaptic organization in *Caenorhabditis elegans*. (2006) J. Biol. 5, 9.1-9.26.

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Schwartz, H. and Horvitz, H.R. (2007) The *C. elegans* protein CEH-30 protects male-specific CEM neurons from apoptosis independently of the Bcl-2 homolog CED-9. Genes & Develop. 21, 3181-3194.

Cancer Investigation, 20(2), 264–275 (2002)

SPECIAL ARTICLE

Malignant Worms: What Cancer Research Can Learn from *C. elegans*

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INTRODUCTION

An important goal in cancer research is to identify and understand the molecular changes that transform normal cells into tumor cells. Ultimately, these insights should translate into opportunities for improved diagnosis and treatment. Over the past two decades, cancer researchers have identified an extensive series of genes that contribute to neoplastic transformation when genetically altered. A detailed understanding of how the normal and mutated gene products function in biological processes should expand the possibilities for therapeutic intervention.

Revealing how a given gene acts amidst the tens of thousands of other genes in the cell remains a challenging task which usually requires a combination of genetic, molecular, biochemical, and cell biological approaches in a number of model systems. Over the past 25 years, genetic studies in the nematode *Caenorhabditis elegans* have made important contributions to our understanding of gene function.

The purpose of this review is to explain the use of the model organism *C. elegans* and its relevance for cancer research. We discuss important contributions in three areas. First, homologs of human oncogenes and tumor suppressors have been found to act in genetic pathways that control well defined biological processes in *C. elegans*. Importantly, the gene networks elucidated in *C. elegans* appear widespread in the animal kingdom and usually are similar to those used in humans. Thus, significant insights have been obtained into the function of human cancer genes by studying their counterparts in the nematode. Second, although tumorigenesis is rarely addressed in *C. elegans*, growth and differentiation processes have been intensely studied. Such studies have identified novel mechanisms and novel genes that have proven or predicted human counterparts and potential relevance in cancer formation. Third, *C. elegans* is being used in screens for therapeutic agents. Although still in the early stages, such screens have enormous potential since oncogenic mutations can be incorporated into the genetic background, which allows a targeted selection of drugs that affect mutant but not wild-type animals.

We will discuss several regulatory pathways that are well established in *C. elegans* developmental studies and indicate the components that have been identified as

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cancer related genes in other systems. Due to the broad area covered, we will emphasize just a few pathways, mention others only in brief and refer to more comprehensive reviews whenever possible. Together, these signal-transduction pathways highlight the contributions of *C. elegans* towards understanding gene function, identifying candidate cancer genes and selecting potential anticancer drugs.

CAENORHABDITIS ELEGANS AS A MODEL ORGANISM

The studies of *C. elegans* stem from a search by Sidney Brenner for an organism in which development and neuronal function could be subjected to genetic analyses (1). Important criteria in choosing *C. elegans* included its relatively simple body plan, ease of cultivation, and genetic tractability. The combination of these features suggested that approaches that had been proven efficient in the studies of bacteria and bacterial viruses could be applied to *C. elegans*.

C. elegans is a small soil nematode; adults are just 1 mm in length and feed on microorganisms. In the lab, thousands of animals can be grown on a single petri dish with *E. coli* bacteria as a food source. Its life cycle is short: in just over three days a fertilized egg develops into an adult hermaphrodite or male. The existence of two different sexes allows for reproduction by either self-fertilization or cross-fertilization, which greatly aids genetic manipulations and screens. For instance, self-fertilization of a single hermaphrodite results in about 300 progeny in three days. If this animal is heterozygous for a recessive mutation, it can produce homozygous progeny without mating. At the same time, the mutant allele can be recovered from the heterozygous siblings, which is particularly useful in the case of lethal and sterile mutations.

The adult hermaphrodite contains only 959 nuclei outside the germline. Despite this simplicity, many different cell types and tissues are present, including muscle, intestine, hypodermis (skin), and neuronal cells. The formation and function of each of these tissues can be studied in the transparent living animals and the function of individual cells can be revealed by laser ablation as well as genetic mutation.

Within 30 years of its initial selection as an animal model, the combination of its features has allowed "the worm" to become the most completely characterized multicellular organism. Concerted efforts of workers in the field have produced milestones that are equally important as they are impressive: the description of the entire somatic cell lineage (2,3), the reconstruction of all neuronal connections from EM images (4), and the first completed DNA sequence of a multicellular organism (5). These and many other accomplishments provide the best validation for the selection of *C. elegans* as an animal model.

GENETIC IDENTIFICATION OF GENE FUNCTION: FORWARD AND REVERSE GENETICS

The most powerful aspect of *C. elegans* research is the animal's genetic tractability. In classic genetic studies, mutants that show defects in a biological process of interest are isolated and studied in order to dissect the process into individual steps, identify the genes involved, and order these genes into pathways. Such studies start with inducing mutations. One of the most potent and the most widely used mutagenic agents is ethylmethanesulphonate (EMS), which induces mostly point mutations (G/C → A/T transitions). The measured frequency of EMS induced mutations affecting an average-size gene is as high as 5×10^{-4} (6). In practical terms, this means that following EMS mutagenesis and self-fertilization of young adult hermaphrodites, frequently a mutant allele can be found in the self-progeny of as few as 1000 F1 animals grown on, for instance, just a hundred petri dishes. Mutations have also been obtained efficiently with various other chemical agents, several radiation methods and, to a lesser extent, transposon induced mutagenesis.

As most known metazoan gene families include a conserved *C. elegans* counterpart, increasingly researchers working with other organisms have found *C. elegans* relatives of their genes of interest. The demand for reverse genetic approaches increased steadily coincident with the progress of the *C. elegans* sequencing project. Reverse genetics starts from a known gene and aims at obtaining a mutant phenotype to reveal its function. Recently, this goal has become feasible due to the development of two novel approaches. The most spectacular method is "RNA-mediated interference" (RNAi) which involves introduction of a double-stranded RNA that includes at least 150 bp of the coding sequence of a particular gene. Although the mechanism of action is not understood as yet, RNAi has been shown to cause a specific loss-of-function phenotype for many of the different genes tested (7–9).

The second reverse genetic approach is selection of a gene knockout. The standard procedure is to screen

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(A)

Gene A	Gene B	
+	+	Wild Type
OFF	+	Phenotype
GF	+	Opposite Phenotype
+	OFF	Opposite Phenotype
OFF	OFF	Opposite Phenotype

Conclusion:

Gene A ⊣ Gene B → Normal Process

(B)

ced-9	*ced-4*	*ced-3*	
+	+	+	Wild Type
OFF	+	+	Extra Cell Death
+	OFF	+	No Cell Death
+	+	OFF	No Cell Death
OFF	OFF	+	No Cell Death
+	GF	+	Extra Cell Death
+	GF	OFF	No Cell Death

Conclusion:

ced-9 ⊣ *ced-4* → *ced-3* → Cell Death

Figure 1. Ordering gene functions into pathways. (A) An example is given of a classic regulatory pathway in which turning genes "on" and "off" determines the outcome of the pathway. Genes that act in this pathway can be defined by mutants with very similar or directly opposite phenotypes. The order of these genes can be established by genetic epistasis analysis. If two genes act in opposite ways, the downstream gene will determine the phenotype in a double mutant combination and this gene is normally inhibited (indicated by the bar in the figure rather than arrowhead) by the gene upstream. If loss-of-function of different genes in the pathway result in similar phenotypes, combinations of loss-of-function (OFF) and gain-of-function (GF) mutations can be used to establish the order of gene activities. (+) represents wild-type gene activity. (B) Example of a switch regulatory pathway for programmed cell death. The *ced-9* gene is required to prevent cells from dying. The *ced-3* and *ced-4* genes act to execute programmed cell death. The double mutant combinations indicate how these may act together in a single regulatory pathway. Alternatively, these genes could act in parallel pathways, but numerous genetic and biochemical experiments strongly support a single linear pathway (12,45).

mutagenized populations for small chromosomal deletions by PCR amplification (10). This method is attractive because it can generate clean genetic null alleles, however, it is still laborious and time consuming. At present, neither reverse genetic method can guarantee success. Although the success rates of RNAi-induced phenotypes are very high, genes with neuronal functions appear relatively resistant. Nevertheless, it will be of great interest to apply these approaches to the *C. elegans* orthologs of human disease genes, especially those implicated in cancer that have not yet been genetically identified in *C. elegans*.

ORDERING GENES INTO PATHWAYS

The goal of genetic analysis is to determine how genes function in biological processes and how genes act together in regulatory pathways or gene networks in vivo. The genetic "logic" used to draw conclusions about the function and order of genes is not always clear to the non-geneticist. We will spend a few sentences below to explain this logic.

In forward genetics, the genes acting in a specific biological process are defined by mutations. For instance, to identify genes that control programmed cell death, one can isolate mutants in which cells fail to die as well as mutants in which too many cells die. Such opposite phenotypes might result from loss-of-function or gain-of-function mutations in the same gene or, alternatively, from mutations in two genes that affect the process in opposite ways. Thus, for conclusions about gene function based on mutant phenotype it is essential to determine the nature of the allele: does it cause complete inactivation (null allele), partial loss of function, gain of function or a novel function? A number of genetic experiments are commonly performed to address these questions (described in Ref. 1). For instance, one should determine whether the mutation is recessive or dominant, as nearly all loss-of function mutations cause recessive phenotypes, whereas gain-of-function and novel-function mutations are usually dominant.

How does one determine the order of gene activities that accomplish a process in vivo? The best indication comes from analysis of the phenotype of double mutant combinations or "genetic epistasis analysis". Genes that affect the same biological process based on their mutant phenotype may act in a single regulatory pathway. If the mutant phenotypes are opposite, genetic epistasis analysis can be used to order the functions of genes with respect to each other. The researcher crosses

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animals that contain single mutations to generate double mutants. If the genes have opposite functions in a single pathway, the phenotype of the double mutant will be similar to one of the single mutants (Fig. 1A). The "epistatic" gene whose mutant phenotype is observed in this analysis can be assigned as the downstream gene in a linear pathway.

As an example, we may consider mutations with opposite effects in programmed cell death (Fig. 1B). One mutation causes extra cell death and thus this gene may normally prevent cell death. Two other mutations prevent cell death, and thus the normal functions of these genes probably are required for the execution of cell death. If the death preventing and death promoting mutations are combined, the double mutant may, for instance, display no cell death. In this case, the death-preventing mutation is epistatic to the death-promoting one. The "lack of cell death" phenotype apparently does not depend on the function of the other gene. Therefore, the gene defined by the death-preventing mutation must act downstream of the other gene if these two genes act in a linear pathway (Fig. 1). However, the two genes could also act in two parallel pathways. The demonstration of direct protein–protein interactions in biochemical studies often provides strong support for a proposed linear pathway.

Genetic epistasis analysis is instrumental in determining the order of gene activities in vivo. As this analysis depends on the creation and combination of mutations, it is most powerful in facile genetic systems such as *C. elegans*. Indeed, the order of gene activities has been established for many signaling pathways in *C. elegans*, frequently also applying to pathways involved in tumorigenesis (see below).

IDENTIFICATION OF FUNCTION OF CANCER-RELATED GENES

A question that immediately follows the identification of a candidate oncogene or tumor suppressor is: what is the normal function of this gene? For many genes, this question has been amazingly difficult to answer. Studies of developmental control mechanisms in *C. elegans* and *Drosophila* have identified many close relatives of mammalian cancer genes. Consequently, the data obtained in these genetically tractable model animals have been tremendously informative in understanding the normal roles that cancer genes play, and in identifying the genetic pathways in which they function.

Below, we have summarized a number of developmental processes in which gene regulatory pathways have been established in *C. elegans*. In each of these pathways, one or more genes resemble human genes with implicated roles in cancer.

THE GENETIC PATHWAY THAT CONTROLS CELL DEATH IS IMPORTANT IN CANCER DEVELOPMENT

Elimination of programmed cell death is an important contributor to cell transformation. The tumor suppressor p53 acts in part by triggering apoptosis in response to DNA damage, and more than 50% of all tumors have lost wild-type p53. In addition, the anti-apoptosis gene *bcl-2* has been found amplified in a subset of tumors (11). Dissecting the molecular mechanisms of apoptosis not only will improve understanding carcinogenesis but also may reveal potential targets for therapeutic intervention.

Programmed cell death is also an important aspect of normal development. Studies in *C. elegans* have provided many fundamental clues to our understanding of how this developmental decision is controlled (12). In *C. elegans*, 131 of the 1090 cells that are generated in the hermaphrodite undergo programmed cell death. Genetic studies have identified specific genes that are required for cell death and others that prevent cells from dying. These genes have been ordered in a genetic pathway that appears conserved between nematodes and humans (Fig. 2). Three genes are required for the execution of all somatic programmed cell deaths: *egl-1*, *ced-3* and *ced-4*. In addition, a single gene, *ced-9*, acts in all cells to prevent programmed cell death. *ced-9* encodes a protein that is similar to the human *bcl-2* oncogene. Human *bcl-2* can partially replace *ced-9* to prevent cell death in *C.*

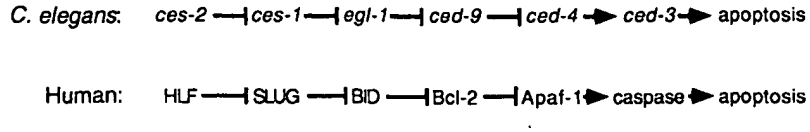

Figure 2. Regulation of programmed cell death (apoptosis) is evolutionarily conserved between *C. elegans* and humans. The different genes involved are discussed in the text.

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elegans (13). *egl-1* encodes another member of the Bcl-2 family, one that consists of only the BH3-domain. *ced-3* is the general executioner of cell death and encodes a cysteine–aspartate specific protease (caspase). Many caspases are expressed in human cells and their role in programmed cell death has largely been revealed through the studies of *ced-3*. Finally, *ced-4* acts genetically upstream of *ced-3* and may promote the cleavage of the caspase to an active form. In human cells, a CED-4 related protein (APAF-1) has been shown to regulate the cleavage of a CED-3 related caspase.

How are specific cells instructed to die? Again mutant analysis in *C. elegans* has provided some clues. The *ces-1* and *ces-2* genes specifically regulate the death of certain neural cells. The CES-2 protein is a transcription factor with a basic-leucine zipper (bZIP) motif that inhibits *ces-1* (Fig. 2; (14)). In turn, CES-1 inhibits the expression of the pro-apoptotic molecule EGL-1. A transcription factor closely related to CES-2, HLF (hepatic leukemia factor), was found to form part of the chimeric oncogene E2A-HLF that can prevent apoptosis of mammalian pre-B lymphocytes (15). E2A-HLF has been suggested to counteract the activity of a human *ces-2* like gene by activating transcription of a *ces-1* like factor with strong antiapoptotic activity (Fig. 2; (16)). Clearly, genetic studies in *C. elegans* have been instrumental to our understanding of the evolutionarily highly conserved process of programmed cell death. This has helped clarify the molecular mechanisms that prevent apoptosis in human malignancy.

CELL FATE DETERMINATION IN *C. ELEGANS* INVOLVES ONCOGENES AND TUMOR SUPPRESSORS

How complex patterns of multiple cell types are formed from cells that were initially indistinguishable is a fundamental question of developmental biology. As in other metazoans, a combination of cell intrinsic and intercellular signaling events determines cell fate in *C. elegans*. The establishment of cell fate patterns has been studied in particular detail for two developmental processes in *C. elegans*: the formation of the hermaphrodite vulva and the specification of the early embryonic progenitor cells. Each of these developmental processes involves a variety of signaling pathways in which homologs of oncogenes and tumor suppressors exert key functions.

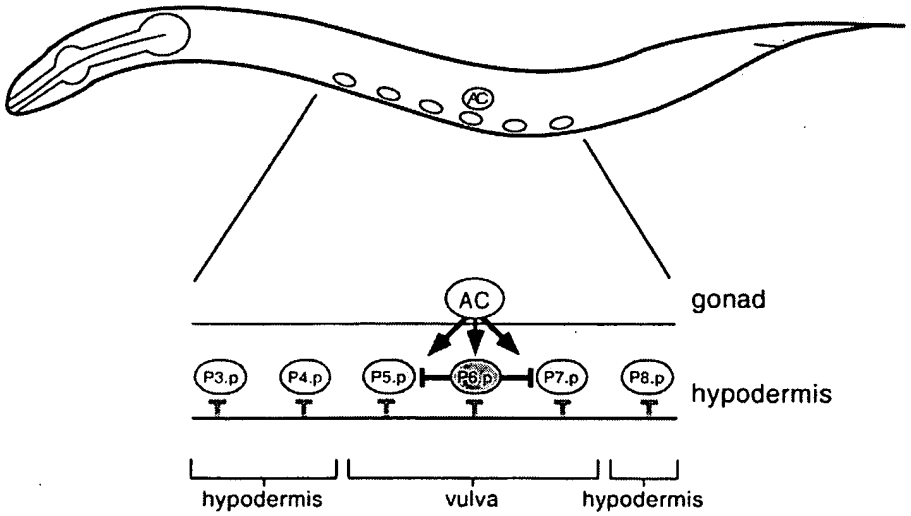

Figure 3. Model for vulval induction in *C. elegans*. The combined activity of at least three distinct regulatory pathways determines the fate of the vulval precursor cells (VPCs: P3.p to P8.p). The inductive signal (arrows) from the anchor cell (AC) involves a Growth Factor/Tyrosine Kinase Receptor/Ras signaling pathway. Lateral inhibition between the VPCs (black bars) involves LIN-12/Notch signaling. The inhibitory signal from the hypodermis (gray inhibitory bar) includes a retinoblastoma-related protein in the VPCs. In the wild-type, the combined descendents of three VPCs (shaded ovals) form the vulva while three other VPCs contribute cells to the hypodermis. Adapted from Ref. (17), see text for further details.

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Cell Fate Specification in Vulval Formation

The hermaphrodite vulva is the structure which connects the reproductive system to the environment and is required for egg laying and sperm entry. Several features have made vulval development a particularly attractive model for studying cell fate determination: the vulva is not required for viability or fertility of the self-fertilizing hermaphrodites and abnormal specification of vulval cell fates is readily detected. An extensive series of mutant analyses and cell ablation experiments have revealed at least three regulatory pathways whose concerted activity determines if cells adopt a vulval fate, which cells will do so and which fate is chosen out of three alternatives (Fig. 3; (17)).

Six ventrally located hypoepidermal cells have equal potential to generate vulval tissue; however, in the wild-type only three of these cells will do so (Fig. 3). The first signaling cascade is initiated by a cell in the gonad, the anchor cell (AC). This cell induces the vulval precursor

Table 1

C. elegans *Genes and Human Homologs Implicated in Cancer*

Cell death (12)	
ced-9	Bcl-2
ces-2	HLF
Vulval induction (17)	
let-23	EGF receptor
let-60	Ras
lin-12	Notch1/Tan1
lin-35	pRb
lin-45	Raf
sli-1	CBL
Wnt/Wingless signaling (25)	
wrm-1/bar-1/hmp-2	β-Catenin
apc-1	APC
pop-1	TCF/LEF
Dauer formation/TGF-β related signaling (46)	
daf-7	TGF-β superfamily
daf-4	Type II receptor
sma-4	DPC-4
Dauer formation/Insulin-related signaling (29,47)	
akt-1	AKT kinase
akt-2	AKT kinase
daf-18	PTEN
Cell proliferation (37,39–41,48,49)	
air-1/air-2	Aurora kinases
cdk-4	Cdk4
cki-1	p21 Family
cyd-1	Cyclin D
cye-1	Cyclin E
lin-35	pRb
mdf-1	MAD1
mdf-2	MAD2

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cell (VPC) that is its nearest neighbor to adopt a primary fate, thereby assuring that the vulva is formed in the correct position with respect to the gonad. In response, the induced VPC uses lateral inhibitory signaling to prevent its own neighbors from adopting a primary fate. As a result, the two neighboring VPCs will adopt a secondary fate. The daughters of these three cells form the vulval structures (Fig. 3). The remaining three VPCs adopt non-vulval, hypodermal fates. The inductive pathway is antagonized by a general inhibitory mechanism that involves two groups of genes know as the synthetic Multivulva (synMuv) genes. Absence of vulval induction results in a vulvaless animal, whereas absence of the general inhibition results in animals with multiple pseudovulvae.

The Inductive Signal of Vulval Fates

Induction by the AC involves an extensive signaling pathway that is now known to be closely related to the growth factor signaling cascades in which the Ras and Raf oncogenes function. The inductive AC signal is an EGF-like growth factor encoded by the gene *lin-3*. The VPCs respond to this factor through LET-23, a tyrosine kinase receptor of the EGF-receptor family (Table 1). The subsequent signaling pathway involves SEM-5 (Grb2), LET-341 (SOS), LET-60 (Ras), LIN-45 (Raf), MEK-2 (MEK), and MPK-1/SUR-1 (MAP kinase) (reviewed in Refs. (17,18)). Eventually, this cascade regulates transcription through phosphorylation of LIN-1 and LIN-31. LIN-1 is an Ets domain transcription factor which suppresses the vulval fate and is negatively regulated by phosphorylation. LIN-31 is a transcription factor of the forkhead family which upon phosphorylation probably switches from a negative regulator of vulval fate to a positive regulator (19).

Importantly, the identification of this signaling pathway in *C. elegans* occurred simultaneously with the identification of growth control genes in human tissue culture cells and genes involved in *Drosophila* eye development. Several of the components such as SEM-5 (Grb2) were first identified in *C. elegans*. In addition, the finding that the *C. elegans* Ras homolog functions in a growth-factor receptor signaling pathway that controls cell fate was an enormously important contribution to the studies of the *ras* oncogene.

Many other genes have been found to regulate the *ras* signal-transduction pathway. Although their discussion goes beyond the scope this review, it is important to mention that several of these genes are presently novel but will likely have mammalian counterparts. Thus, as soon as such mammalian genes are identified a candidate function is already available. Some of these genes may have roles in carcinogenesis. For example, mutations in the gene *sli-1* were found to suppress the vulvaless phenotype caused by a *let-23* mutation, indicating that *sli-1* normally functions to downregulate *let-23* signaling (20). Interestingly, the *sli-1* gene product is similar to the CBL oncogene product. In agreement with the *C. elegans* data, recent studies have determined that the wild-type, but not the oncogenic, form of CBL acts as an adaptor protein that targets the EGF receptor for proteolytic degradation (21).

Lateral Inhibition

The lateral inhibition between VPCs requires the gene *lin-12* which encodes a transmembrane signaling molecule similar to Notch in *Drosophila* and Notch 1, 2 and 3 in Humans. Human Notch has been implicated in T lymphoblastic leukemia, based on observed translocations that affect the Notch1/TAN-1 transcript (22). Upstream (ligand) and downstream signaling molecules of LIN-12/Notch were not discovered in studies of the VPCs, but they have been identified as components of a *lin-12*-mediated pathway that acts at an earlier time of development. Importantly, an activated form of another Notch-related gene, *glp-1*, causes an extensive germline tumor in *C. elegans* (reviewed in Ref. (23)). A genetic pathway has been established in which *glp-1* negatively regulates the genes *gld-1* and *gld-2*, whose inactivation can cause a similar germline tumor. The *glp-1* pathway appears to regulate the switch between mitotic and meiotic division in the germ line, so that failure to undergo this transition likely results in continued mitotic divisions and a tumorous phenotype. It will be interesting to see whether similar mechanisms and components of the *glp-1/lin-12*/Notch signaling pathways play a role in germline tumors in humans.

General Inhibition of Vulval Fates

General inhibition of vulval fates by the hypodermis is dependent on two groups of genes, class A and class B genes. Single mutations in class A or class B genes do not confer a vulval phenotype. However, double mutants containing both class A and class B mutations are Multivulva. Such interactions, which require mutations in independent genes to obtain a detectable phenotype, often reveal redundancies and are referred to as "synthetic". Therefore the class A and B genes are named synthetic Multivulva (synMuv) genes. Based on

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genetic epistasis studies, the synMuv genes appear to antagonize a ligand independent activity of the EGF-R/Ras pathway. Four class A and ten class B genes have currently been identified. All cloned class A members and several of the class B genes appear to encode novel proteins. However, the *lin-35* class B product is similar to the retinoblastoma-susceptibility protein pRb. In addition, another class B gene, *lin-53*, encodes a protein similar to RbAp48 that interacts with pRb. It will be of great interest to determine whether homologs of the other synMuv genes act as tumor-suppressor genes in humans.

OTHER GENES INVOLVED IN *C. ELEGANS* DEVELOPMENT AND HUMAN CANCER

Wnt/Wingless Signaling

Various other signaling pathways have been identified that regulate aspects of *C. elegans* development. Again, several of these pathways contain orthologs of human oncogenes or tumor suppressors. For example, Wnt/Wingless signaling controls the asymmetric division of a number of different cell types during early embryonic development as well as larval development in *C. elegans*. The basic components of the Wnt/Wingless pathway were first identified in other systems such as *Drosophila* and *Xenopus*. Homologs of the "adenomatous polyposis coli" (APC) tumor-suppressor and the β-catenin oncogene have been shown to play important roles in this pathway (24). Recent studies have revealed novel aspects of this pathway in *C. elegans*: several of the genes have been found to act in ways opposite to their counterparts in vertebrates, components of the pathway have been found to regulate the position of the mitotic spindle, and connections have been identified between Wnt and MAP kinase pathways (25). Although it is currently unclear to what extent these aspects are evolutionarily conserved, they are likely to represent variations on themes that are also used in mammals within the context of specific cells or promoters. Therefore, it will be of great interest to examine whether any of the newly discovered functions of Wnt signaling in *C. elegans* are conserved in humans and specifically altered in cancer cells.

TGF-β- and Insulin-Signaling

Several other important pathways regulate the execution of an alternative developmental program. If the food supply becomes limiting or the population too dense, young larvae arrest their growth and become "dauer" larvae. Dauer larvae do not eat, have thick protective cuticles and are specialized for survival during harsh conditions. Genetic and molecular characterizations have revealed that dauer formation is controlled by several parallel pathways that are related to TGF-β- and insulin-signaling pathways in mammals (Table 1; (26)). The analysis of these pathways in *C. elegans* has helped to rapidly designate functions to newly identified human tumor suppressors. Examples include the tumor suppressor DPC4, deleted in pancreatic cancer, which is related to SMAD transcription factors that act downstream of the TGF-β receptors (27,28). Another example is PTEN, a recently identified tumor suppressor gene that acts in insulin-related signaling and dauer formation in *C. elegans* (29). It is of great interest to examine whether homologs of other genes in these *C. elegans* pathways, such as the forkhead transcription factor *daf-16*, play roles in human cancer.

Cell-Division Control in *C. elegans*

The process of cell division itself has yet to become a well-established field of study in *C. elegans*. Nevertheless, this animal is ideally suited for such studies: the timing of every somatic cell division is known and all divisions can be followed in vivo, due to the animal's transparency. Several areas of cell-cycle control are even more tractable in *C. elegans* than in yeast, the traditional genetic system for cell-cycle studies. Such processes are more readily visible in the worm, are specific for multicellular organisms or involve genes that are not conserved in single cell eukaryotes. Examples include chromosome segregation and cytokinesis, checkpoint mechanisms that trigger apoptosis and developmental control of cell division. Below, we summarize some cell-cycle research in *C. elegans* that appears the most promising and important to the cancer investigator.

A few interesting cell-cycle mutants were identified in early genetic *C. elegans* screens. For instance, *lin-19/cul-1* mutants display extra divisions of many different cell types during larval development (30). The *cul-1* gene has become the founding member of a conserved family of "cullins", which are accessory factors in the targeting of proteins for ubiquitin-dependent degradation. The molecular mechanisms of this proteolysis process have largely been revealed through studies in yeast and *Xenopus*. Interestingly, the von Hippel–Lindau tumor suppressor protein (VHL) forms part of a complex that includes a cullin family member, Cul2 (reviewed in Ref. (31)). This complex is involved in degradation of the "hypoxia-inducible factor" (HIF), which normally

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promotes blood vessel formation under conditions of low oxygen. *C. elegans* may provide a genetic system for analysis of VHL function as candidate VHL, Cul2 and HIF homologs are all present in the worm (5,32).

The accurate segregation of chromosomes during M phase is essential for the maintenance of genomic stability. Condensed chromosomes and mitotic spindles are visible with the light microscope in *C. elegans*. Moreover, the actual cell division in *C. elegans* occurs through a cytokinesis process that morphologically resembles that in mammals. Due to these features, *C. elegans* is rapidly becoming an important model for chromosome separation, mitotic spindle function and cytokinesis (33). Genetic screens and RNA interference experiments have recently defined many candidate components of these processes (34,35) and new molecules are likely to be found. An example is the *lin-5* gene which encodes a novel protein required for segregating chromosomes and preventing exit from mitosis when chromosome segregation is incomplete (36). In addition, genes previously implicated in accurate chromosome segregation, such as components of the spindle-assembly checkpoint, are being studied with the help of reverse genetic approaches (37).

In response to DNA damage, checkpoint mechanisms halt the cell cycle to create time for repair. In addition, the cell's suicide program may be activated if the damage is too extensive. A recent study has demonstrated that DNA damage can trigger apoptosis of germ cells in *C. elegans* (38). The core apoptosis machinery is involved in this response in addition to checkpoint genes that include a homolog of the fission yeast *rad1*$^+$ gene. These results indicate that genetics in *C. elegans* should be useful in identifying mechanisms that activate the cell-death machinery in response to DNA lesions. Cancer cells are selected to escape such mechanisms.

To coordinate cell division with growth and differentiation, extracellular signals communicate with the cell-cycle machinery during G1 phase. Our recent results indicate that G1 control in *C. elegans* follows molecular mechanisms closely related to those used in mammalian cells. G1 progression depends on positive regulators homologous to the D-type cyclins and cdk4/6 kinases ((39); Boxem and van den Heuvel, unpublished observations). Moreover, it involves negative regulators related to the retinoblastoma (Rb) protein and the p21/p27 CIP/KIP family of CDK inhibitors ((40,41); Boxem and van den Heuvel, unpublished observations). Moreover, we have isolated mutations that disturb cell division in specific lineages and developmental stages, likely defining genes that act upstream of the cell-cycle machinery. Several of these mutations allow a subset of cells to divide during the second larval stage. Interestingly, the genes involved appear to define a pathway for cell lineage dependent transcriptional regulation of a CIP/KIP CDK inhibitor (Saito and van den Heuvel, unpublished observations). These results start to reveal aspects of developmental control of cell division. At present, such controls are poorly understood despite their importance in normal development as well as cancer.

THE USE OF *C. ELEGANS* IN DRUG DISCOVERY

We have described above how mutant analysis in *C. elegans* has helped the identification of in vivo functions and signaling pathways of human cancer genes. The conservation of these pathways opens the possibility to use *C. elegans* in the discovery of anticancer therapeutics (42). Previously known drugs have been commonly applied in *C. elegans* studies (43). Such studies have helped dissect the processes in which the drugs interfere and have led to identification of drug targets. A particularly attractive example involves the analysis of farnesyltransferase inhibitors for their effect on activated Ras. Because of the importance of farnesylation in Ras localization and cell transformation, farnesyltransferase inhibitors have been developed as candidate chemotherapeutic agents. Hara and Han showed that such inhibitors specifically revert the Multivulva phenotype (see above) caused by a *let-60* Ras gain-of-function mutation (44). Thus, suppression of a specific and selectable phenotype appears possible in *C. elegans* drug screens.

Selecting compounds for activity in *C. elegans* has the advantage of directly testing effects in the context of an entire animal. This will create a bias towards bioactive compounds that are able to penetrate cells, are metabolically stable and have relatively specific effects. The assays can be performed in small-scale cultures using high throughput methods, typically microtiter plates, thus relatively inexpensive large-scale selections are possible.

An equally attractive aspect of drug selection in the nematode is the possibility to take advantage of genetic methods (42,43). Genetics can help the drug discovery process at several different steps. First, the effects of mutations are informative, as a mutation eliminates the function of one and only one protein, similar to the perfect therapeutic compound. Second, selection of bioactive compounds can be made highly efficient by using specific genetic backgrounds in which, for

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instance, the desired drug will alleviate a lethal or sterile phenotype. Third, screens can be performed for their effects on an entire pathway, rather than a specific molecule. Fourth, genetics can be used to establish the target of a bioactive compound once a compound has been identified (43). Subsequently, this component can be modified for optimal effects on a human homolog. Finally, genetic manipulation allows an alternative strategy that starts with the introduction of a human gene, possibly but not necessarily replacing a *C. elegans* gene, and screening for modification of specific effects that result from expression of this gene. Although drugs selected by any of these methods have not been reported as yet, several pharmaceutical companies have initiated studies based on the application of *C. elegans* in the discovery of lead therapeutic compounds.

SUMMARY AND CONCLUSIONS

Developmental processes in the nematode *C. elegans* are controlled by pathways of gene functions that are analogous to those used in mammals. Hence, genetic studies in *C. elegans* have helped build the frameworks for these regulatory pathways. Many homologs of human genes that are targets for mutation in cancer have been found to function at distinct steps within such genetic pathways. This way, studies in *C. elegans* have provided important clues about the functions of human oncogenes and tumor suppressors.

Understanding how human cancer genes function and act in signaling cascades is of great importance. This information reveals what kind of molecular changes contribute to the process of cell transformation. Moreover, additional candidate oncogenes and tumor suppressors may be revealed by identifying the functional partners of genes with an established role in cancer. Furthermore, identifying a cascade of gene functions increases the number of potential targets for therapeutic intervention, as blocking either one of multiple genes may interfere with signal transduction through the pathway.

Simultaneous approaches in a number of different model systems act synergistically in solving pathways of gene functions. By using multiple models, the field takes advantage of the strengths of each system and circumvents its limitations. As one of the most powerful genetic animal systems, *C. elegans* will continue to reveal new mammalian signaling components. In addition, now that the *C. elegans* genome sequence has been completed, an increasing number of researchers are likely to discover homologs of human disease genes in the nematode and to analyze gene function in the worm model. Combined with the great potential of this animal in drug screens, it is simple to predict that *C. elegans* will worm its way deeper and deeper into cancer research.

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